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New record of *Aplosporella javeedii* on five hosts in China based on multi-gene analysis and morphology

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Abstract —*Aplosporella javeedii* (*Aplosporellaceae*) was collected from branch cankers in China, identified by multigene phylogenetic analyses and morphological characters, described, and illustrated. This species is newly recorded for China, and the five tree species (*Albizia julibrissin*, *Broussonetia papyrifera*, *Gleditsia sinensis*, *Juniperus chinensis*, and *Styphnolobium japonicum*) represent new host records for *A. javeedii*.

Key words — *Ascomycota*, *Botryosphaeriales*, phylogeny, taxonomy

Introduction

Aplosporella Speg. is characterized by solitary, globose, unilocular conidiomata in malt extract agar (MEA) and multi-locular branched conidiomata with a single ostiole and brown aseptate conidia.

Jami et al. (2014) described *Aplosporella javeedii* as an endophyte from healthy wood sections of *Celtis africana* Burm. f. (*Cannabaceae*) and *Searsia lancea* (L. f.) F.A. Barkley (*Anacardiaceae*) in South Africa. During our recent taxonomic study of botryosphaeriaceous fungi associated with dieback diseases in China, isolates from five different Chinese hosts were identified as *Aplosporella javeedii* based on morphological and multi-gene phylogenetic analyses. These represent the first records of *A. javeedii* from China.

Materials & methods

Collection of samples and isolates. — Six *Aplosporella* isolates were collected from five hosts: *Albizia julibrissin* Durazz. (*Fabaceae*), *Broussonetia papyrifera* (L.) Vent.

(*Moraceae*), *Gleditsia sinensis* Lam. (*Fabaceae*), *Juniperus chinensis* L. (*Cupressaceae*), and *Styphnolobium japonicum* (L.) Schott [≡ *Sophora japonica* L.] (*Fabaceae*). Isolations were made directly from conidiomata on the host and single-spore purification according to a protocol slightly modified from Fan et al. (2014). Specimens are deposited in the Museum of Beijing Forestry University, Beijing, China (BJFC), and single-spore cultures are maintained in the China Forestry Culture Collection Center, Beijing, China (CFCC).

Morphological studies. — Specimens observed on branch tissues consisted of size of fruiting bodies and spores. More than 20 fruiting bodies were sectioned, and 50 randomly selected spores were measured using a Leica light microscope (DM 750). Cultural characteristics of isolates incubated on MEA in the dark at 25°C were recorded. These included colony characters and pigment production, at 3, 7, and 30 days.

DNA extraction, PCR amplification, and sequencing. — Genomic DNA was extracted from pure cultures grown in MEA with cellophane at 25°C for 2 weeks. Genomic DNA was extracted using a modified CTAB method (Doyle & Doyle 1990). DNA concentrations were estimated by electrophoresis in 1% agarose gels and NanoDrop™ 2000 (Thermo, USA) according to Desjardins et al. (2009). The Beijing Genomics Institute sequenced the DNA. Amplifications were primed by ITS1 & ITS4 (nITS region; White et al. 1990), NL1 & NL4 (LSU region; O'Donnell 1993), and EF1-688F & EF1-1251R (EF-1α region; Alves et al. 2008). PCR amplification products were estimated visually by electrophoresis in 2% agarose gels. DNA sequencing was performed using an ABI PRISM® 3730XL DNA Analyzer with BigDye® Terminator Kit v.3.1 (Invitrogen) at the Shanghai Invitrogen Biological Technology Company Limited (Beijing, China).

DNA sequence analysis. —DNA sequences generated by forward and reverse primers were used to obtain consensus sequences using SeqMan v.7.1.0 in the DNAStar Lasergene Core Suite software (DNAStar Inc., Madison, WI, USA). Sequences were aligned using MAFFT v.6 (Katoh & Toh 2010) and edited manually using MEGA 6 (Tamura et al. 2013). A partition homogeneity test with heuristic search and 1000 homogeneity was performed using PAUP v.4.0b10 to test the discrepancy among the three loci in reconstructing phylogenetic trees. Phylogenetic analysis was run using PAUP v.4.0b10 for maximum parsimony (MP) analysis (Swofford 2003), MrBayes v.3.1.2 for Bayesian analysis (Ronquist & Huelsenbeck 2003), and RAxML v.7.2.8 for maximum likelihood (ML) analysis (Stamatakis 2006). A phylogeny using *Aplosporella javeedii* sequences from the current study and other reference sequences in GenBank was generated based on the multilocus alignment (ITS, LSU, EF-1α). *Fusicladium effusum* STE-U 4525 and *Fusicladium oleagineum* CBS 113427 were selected as outgroup (Slippers et al. 2013). Trees were visualized using FigTree v.1.3.1 (Rambaut & Drummond 2010).

MP analysis was run using a tree-bisection and reconnection branch-swapping algorithm with heuristic search option of 1000 random-addition sequences. Zero length branches were collapsed and all equally parsimonious trees were saved. Other calculated parsimony scores calculated were tree length (TL), consistency index (CI), retention index (RI) and rescaled consistency (RC). ML analysis was also performed using a GTR model of site substitution, including estimation of gamma-distributed rate heterogeneity and a proportion of invariant sites (Stamatakis 2006). The MP and ML support values were evaluated with a bootstrapping method of 1000 replicates (Hillis & Bull 1993). Bayesian inference was calculated using a Markov Chain Monte Carlo (MCMC) algorithm with Bayesian posterior probabilities (Rannala & Yang 1996). A nucleotide substitution model was estimated by MrModeltest v.2.3 (Posada & Crandall 1998), and a weighted Bayesian analysis was considered. Two MCMC chains were run from random trees for 1,000,000 generations, and trees were sampled every 100th generation, resulting in 10,000 total trees. The first 25% of trees were discarded as the burn-in phase of each analysis and the posterior probabilities (PP) were calculated using the remaining 7500 trees.

Sequence data is deposited in GenBank, and the accession numbers are listed in Table 1. The multilocus sequences alignment file is deposited in TreeBASE (www.treebase.org) as accession S16645.

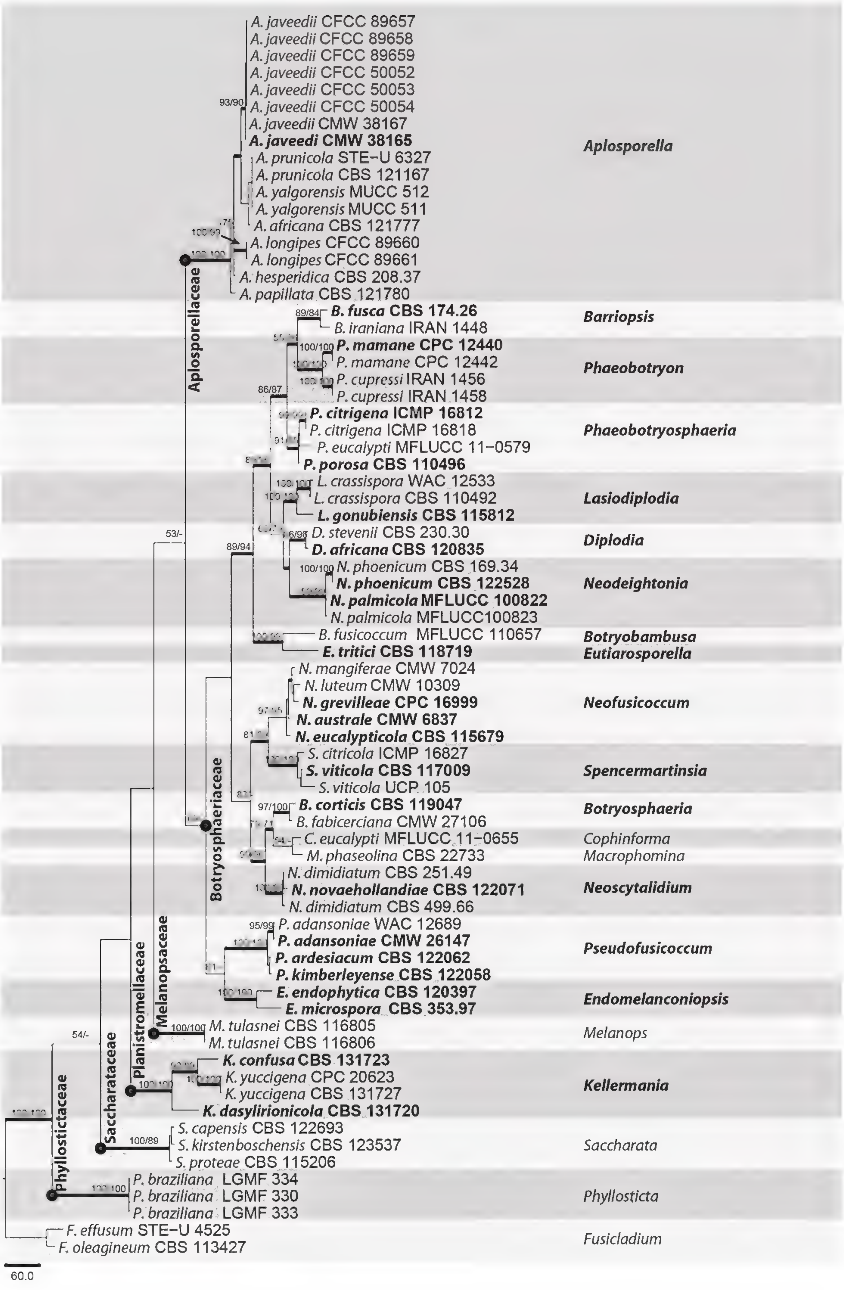
Table 1 – Newly sequenced strains of *Aplosporella javeedii* used in the phylogenetic analyses.

Specimen	Host	Strain	GenBank accession numbers		
			ITS	LSU	EF-1α
BJFC-S867	*Albizia*	CFCC 89657	KM030579	KM030586	KM030593
BJFC-S884	*Broussonetia*	CFCC 89658	KM030580	KM030587	KM030594
BJFC-S920	*Albizia*	CFCC 89659	KM030581	KM030588	KM030595
BJFC-S1022	*Gleditsia*	CFCC 50052	KP208838	KP208841	KP208844
BJFC-S1042	*Styphnolobium*	CFCC 50053	KP208839	KP208842	KP208845
BJFC-S1044	*Juniperus*	CFCC 50054	KP208840	KP208843	KP208846

Phylogeny

The combined multi-gene region dataset included sequences from 71 ingroup strains representing 48 species in *Botryosphaeriales*. A partition homogeneity test was not significant (P = 0.145) indicating that the individual datasets were congruent and produced trees with the same topologies. The dataset had an aligned length of 1468 characters of which 772 characters are constant, 74 are variable parsimony-uninformative, and 622 are parsimony-informative. Maximum parsimony analysis yielded three equally parsimonious trees (TL = 2540, CI = 0.490, RI = 0.809, RC = 0.396). The ML analysis produced the same topology as the MP tree, with Bayesian analysis producing the same topology, with an average standard deviation of split frequencies = 0.007233.

The phylogeny inferred from ITS, LSU, and EF-1α sequences (Fig. 1) indicated six major clades: *Aplosporellaceae*, *Botryosphaeriaceae*, *Melanopsaceae*,


A. javeedii CFCC 89657
A. javeedii CFCC 89658
A. javeedii CFCC 89659
A. javeedii CFCC 50052
A. javeedii CFCC 50053
A. javeedii CFCC 50054
A. javeedii CMW 38167
A. javeedi CMW 38165
A. prunicola STE-U 6327
A. prunicola CBS 121167
A. yalgorensis MUCC 512
A. yalgorensis MUCC 511
A. africana CBS 121777
A. longipes CFCC 89660
A. longipes CFCC 89661
A. hesperidica CBS 208.37
A. papillata CBS 121780
93/90
100/99
Aplosporella
Aplosporellaceae
B. fusca CBS 174.26
B. iraniana IRAN 1448
89/84
Barriopsis
P. mamane CPC 12440
P. mamane CPC 12442
P. cupressi IRAN 1456
P. cupressi IRAN 1458
100/100
Phaeobotryon
86/87
P. citrigena ICMP 16812
P. citrigena ICMP 16818
P. eucalypti MFLUCC 11-0579
P. porosa CBS 110496
Phaeobotryosphaeria
L. crassispora WAC 12533
L. crassispora CBS 110492
L. gonubiensis CBS 115812
Lasiodiplodia
53/-
89/94
D. stevenii CBS 230.30
D. africana CBS 120835
Diplodia
N. phoenicum CBS 169.34
N. phoenicum CBS 122528
N. palmicola MFLUCC 100822
N. palmicola MFLUCC100823
100/100
Neodeightonia
B. fusicoccum MFLUCC 110657
E. tritici CBS 118719
Botryobambusa
Eutiarosporella
N. mangiferae CMW 7024
N. luteum CMW 10309
N. grevilleae CPC 16999
N. australe CMW 6837
N. eucalypticola CBS 115679
Neofusicoccum
S. citricola ICMP 16827
S. viticola CBS 117009
S. viticola UCP 105
Spencermartinsia
B. corticis CBS 119047
B. fabicerciana CMW 27106
97/100
Botryosphaeria
C. eucalypti MFLUCC 11-0655
M. phaseolina CBS 22733
Cophinforma
Macrophomina
Botryosphaeriaceae
N. dimidiatum CBS 251.49
N. novaehollandiae CBS 122071
N. dimidiatum CBS 499.66
Neoscytalidium
P. adansoniae WAC 12689
P. adansoniae CMW 26147
P. ardesiacum CBS 122062
P. kimberleyense CBS 122058
95/99
Pseudofusicoccum
E. endophytica CBS 120397
E. microspora CBS 353.97
Endomelanconiopsis
M. tulasnei CBS 116805
M. tulasnei CBS 116806
100/100
Melanops
Melanopsaceae
54/-
K. confusa CBS 131723
K. yuccigena CPC 20623
K. yuccigena CBS 131727
K. dasylirionicola CBS 131720
Kellermania
Planistromellaceae
S. capensis CBS 122693
S. kirstenboschensis CBS 123537
S. proteae CBS 115206
100/89
Saccharata
Saccharataceae
P. braziliana LGMF 334
P. braziliana LGMF 330
P. braziliana LGMF 333
Phyllosticta
Phyllostictaceae
F. effusum STE-U 4525
F. oleagineum CBS 113427
Fusicladium
60.0

Phyllostictaceae, *Planistromellaceae*, and *Saccharataceae*, representing six families in *Botryosphaeriales*. The sequences from our six Chinese collections clustered within *Aplosporellaceae*, where they formed a highly supported clade (MP = 93; ML = 90; BBP = 0.99) with the South African holotype and paratype collections of *Aplosporella javeedii*.

Taxonomy

Aplosporella javeedii Jami, Gryzenh., Slippers & M.J. Wingf., Fungal Biology 118: 174. 2014. FIG. 2

SEXUAL STATE: unknown.

ASEXUAL STATE: Conidiomata immersed in bark, erumpent from bark surface, separate, discoid, dark-brown to black, multi-locular, ≤1.5 mm diam, wall consisting of 4–10 layers, thick outer layers composed of dark-brown textura angularis, becoming hyaline and thin-walled towards the inner region. Disc brown to black, circular to ovoid, (0.43–)0.48–0.66(–0.75) mm (average = 0.58 mm, n = 20), with 1–5 ostioles per disc. Ostioles multiple, conspicuous, level with the disc surface, sometimes covered below disc by lighter entostroma, (52.8–)61.0–85.2(–89.9) µm (average = 77.6 µm, n = 20) in diam. Locules multiple, irregularly arranged, subdivided frequently by invaginations with common walls, (0.84–)0.93–1.40(–1.52) mm (average = 1.26 mm, n = 20) in diam. Paraphyses hyaline, smooth-walled, 31.4–80.1 µm long, 3.1–5.5 µm wide. Conidiogenous cells formed from the cells lining the inner walls of the locules, hyaline, smooth, inconspicuous. Conidia aseptate, ellipsoid to oblong, smooth, ends rounded, initial hyaline, becoming brown when mature, (18.8–)19.5–24.6(–25.3) × (8.3–)9.6–11.7(–12.9) µm (average = 22.8 × 10.6 µm, n = 50). Cultures in PDA white, uniform, felty with abundant aerial mycelium. Cultures in MEA white at first, then grey-olivaceous, similar to reverse side after 7–10 days. Colony uniform with appressed aerial mycelium. After 3 weeks, conidiomata solitary, globose, unilocular, immersed to semi-immersed on MEA.

MATERIAL EXAMINED: CHINA, GANSU PROVINCE, Qingyang City, municipal government, 35°42′31.02″N 107°38′24.94″E, elev. 1400 m asl, on infected branches of *Albizia julibrissin*, 14 July 2013, X.L. Fan (BJFC-S867; culture CFCC 89657);

FIGURE 1. Phylogram of the combined genes of ITS, LSU, and EF-1α based on MP, ML, and Bayesian analyses of botryosphaerialean taxa. Numbers above the branches indicate bootstrap values. Thickened branches indicate PP > 0.95 from the Bayesian inferences. Scale bar = 60 nucleotide substitutions. The newly sequenced Chinese collections of *Aplosporella javeedii* (designated by CFCC #s) are the top six sequences of the tree. Ex-holotype sequences are in bold.

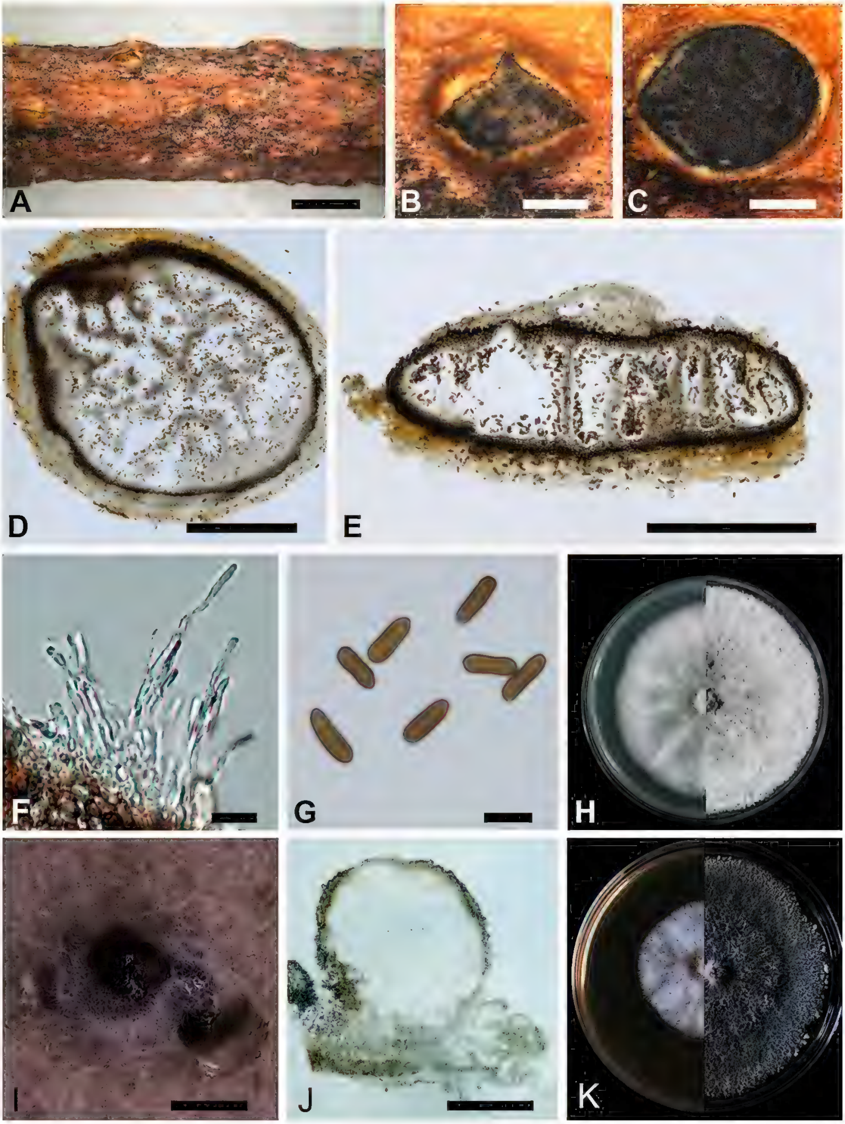


FIGURE 2. *Aplosporella javeedii* (BJFC-S867). A: Habit of conidiomata on a twig. B: Multiple ostioles on the discs of the conidiomata. C, D: Transverse sections through the conidiomata. E: Longitudinal sections through the conidiomata. F: Paraphyses and conidiogenous cells. G: Conidia. H: Colonies on PDA at 3 days (left) and 30 days (right). I: Conidiomata on MEA. J: Transverse sections through the conidiomata on MEA. K: Colonies on MEA at 3 days (left) and 30 days (right). Scale bars: A = 2 mm; B–E, J = 0.5 mm; F, G = 20 µm; I = 1 mm.

Qingyang City, Heshui County, 35°46′51.46″N 107°59′43.42″E, elev. 1291 m asl, on infected branches of *A. julibrissin*, 13 July 2013, X.L. Fan (BJFC-S920; culture CFCC 89659); Tianshui City, Baozi Valley, 34°34′00.21″N 105°42′21.94″E, elev. 1007 m asl, on infected stems of *Broussonetia papyrifera*, 17 July 2013, X.L. Fan (BJFC-S884; culture CFCC 89658). SHANXI PROVINCE, Datong City, Children's Park, 40°04′44.05″N 113°16′42.89″E, elev. 1051 m asl, on infected branches of *Gleditsia sinensis*, 14 April 2014, X.L. Fan & B. Cao (BJFC-S1022; culture CFCC 50052); Jinzhong City, Jinshang Park, 37°43′07.26″N 112°43′52.00″E, elev. 818 m asl, on infected branches of *Styphnolobium japonicum* [≡ *Sophora japonica*], 17 April 2014, X.L. Fan & B. Cao (BJFC-S1042; culture CFCC 50053); Jinzhong City, Jinshang Park, 37°43′08.11″N 112°43′52.20″E, elev. 809 m asl, on infected branches of *Juniperus chinensis*, 17 April 2014, X.L. Fan & B. Cao (BJFC-S1044; culture CFCC 50054).

HABITAT/DISTRIBUTION: Known from *Albizia julibrissin*, *Broussonetia papyrifera*, *Gleditsia sinensis*, *Juniperus chinensis*, and *Styphnolobium japonicum* in China and from *Celtis africana* and *Searsia lancea* in South Africa.

Discussion

Our six Chinese botryosphaeriaceous strains are identified as *Aplosporella javeedii* based on morphological observations and multi-gene phylogenetic analyses. Morphologically, they are characterized by solitary globose unilocular conidiomata on MEA and erumpent discoid conidiomata, multiple ostioles in branches with ellipsoid to oblong aseptate conidia averaging 22.8 × 10.6 µm.

Aplosporella javeedii was previously reported as an endophyte from healthy trees of two dicotyledonous host species in South Africa (Jami et al. 2014). Our Chinese records extend the host range to two additional dicotyledonous families, and also to a coniferous host; moreover, our specimens were collected from branch cankers, raising the question whether this species may have some pathogenic potential. Slippers & Wingfield (2007) suggested that botryosphaeriaceous fungi might shift slightly from endophytic to pathogenic habit when hosts are subjected to stress.

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